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·Original Article·

Endogenous level of TIGAR in brain is associated with vulnerability of neurons to ischemic injury

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ABSTRACT

In previous studies, we showed that TP53-induced glycolysis and apoptosis regulator (TIGAR) protects neurons against ischemic brain injury. In the present study, we investigated the developmental changes of TIGAR level in mouse brain and the correlation of TIGAR expression with the vulnerability of neurons to ischemic injury. We found that the TIGAR level was high in the embryonic stage, dropped at birth, partially recovered in the early postnatal period, and then continued to decline to a lower level in early adult and aged mice. The TIGAR expression was higher after ischemia/reperfusion in mouse brain 8 and 12 weeks after birth. Four-week-old mice had smaller infarct volumes, lower neurological scores, and lower mortality rates after ischemia than 8- and 12-week-old mice. TIGAR expression also increased in response to oxygen glucose deprivation (OGD)/reoxygenation insult or H_2O_2 treatment in cultured primary neurons from different embryonic stages (E16 and E20). The neurons cultured from the early embryonic period had a greater resistance to OGD and oxidative insult. Higher TIGAR levels correlated with higher pentose phosphate pathway activity and less oxidative stress. Older mice and more mature neurons had more severe DNA and mitochondrial damage than younger mice and less mature neurons in response to ischemia/reperfusion or OGD/reoxygenation insult. Supplementation of cultured neurons with nicotinamide adenine dinuclectide phosphate (NADPH) significantly reduced ischemic injury. These results suggest that TIGAR expression changes during development and its expression level may be correlated with the vulnerability of neurons to ischemic injury.

Keywords: TIGAR; NADPH; ischemia; OGD; H_2O_2

INTRODUCTION

Ischemic stroke results from a transient or permanent reduction in cerebral blood flow restricted to the territory of a major brain artery. The oxidative stress associated with excessive production of reactive oxygen species (ROS) is a fundamental mechanism of brain damage in stroke[1-3]. The vulnerability of neurons to ischemic injury increases with age[4], and it has also been reported that neonatal mice are more prone to ROS-induced damage[5,6]. Neonatal stroke leads to morbidity and severe long-term neurological and

cognitive deficits, including cerebral palsy. In patients with ischemic stroke, older patients experience more severe brain damage and have a higher incidence of mortality and disability than younger patients[7]. The mechanisms underlying age-mediated susceptibility to ischemic injury in neonatal, mature, and aged mice are not fully understood. Mitochondrial integrity is known to decline as a function of age, thus increasing oxidative stress[8-10], while other deleterious changes associated with aging include changes in gene expression and the signaling pathways involved in neuronal survival [11,12].

TP53-induced glycolysis and apoptosis regulator (TIGAR) has similarities to the bisphosphatase domain of PFK-2/FBPase-2, a bifunctional enzyme with both kinase and bisphosphatase activities[13]. PFK-2/FBPase-2 regulates both the synthesis (through PFK-2) and the degradation (through FBPase-2) of intracellular fructose-2,6-bisphosphate (Fru-2,6-P2), a potent positive allosteric regulator of 6-phosphofructo-1-kinase (PFK-1), which is one of the key regulators of glycolysis[14]. Therefore, TIGAR can block glycolysis by reducing the level of Fru-2,6-P2. Our previous studies showed that TIGAR also upregulates the expression of G6PD (glucose-6-phosphate dehydrogenase), a rate-limiting enzyme in the pentose phosphate pathway (PPP)[15], such that TIGAR is able to increase PPP flow. PPP is the major source of nicotinamide adenine dinucleotide phosphate (NADPH) and ribose phosphate. NADPH is important for maintaining the levels of reduced glutathione (GSH)[11,16], which is critical for ROS metabolism. A previous study reported that TIGAR protects neurons against ischemic injury by promoting PPP flow[15]. In the present study, we sought to determine the developmental changes of TIGAR protein levels and investigate whether they are correlated with the vulnerability of neurons to ischemic injury.

MATERIALS AND METHODS

Model of Focal Ischemia

Male ICR mice (1, 2, and 3 months old) were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). All animals were used in accordance with the Institutional Guidelines for Animal Use and Care and the study protocol was approved by the Ethical Committee of Soochow University. Middle cerebral artery occlusion (MCAO) surgery was performed as described previously[17,18]. Briefly, mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium. For focal cerebral ischemia, a silicone-coated nylon (6-0) monofilament (Doccol Corp., Redlands, CA) was inserted into the exposed right internal carotid artery and removed 120 min after occlusion. Cerebral blood flow was monitored (LDF ML191 Laser Doppler Blood Flow Meter, Australia) and only mice with 90% reduction of blood flow during MCAO and 85%–95% recovery of blood flow during reperfusion were used for further experiments. Sham-operated mice underwent identical surgery but the monofilament was not inserted. Body temperature was maintained at 37 ± 0.5°C using a heating blanket during surgery. Mice were sacrificed at the indicated times after ischemia/reperfusion.

Assessment of Neurological Score

Twenty-four hours after ischemia/reperfusion, neurological status was assessed using a 5-point rating scale[18]. Deficits were evaluated in a blinded manner using the following scale: 0, normal motor function; 1, flexion of torso and contralateral forelimb when lifted by the tail; 2, circling to the contralateral side when held by the tail on a flat surface but normal posture at rest; 3, leaning to the contralateral side at rest; 4, no spontaneous motor activity.

Assessment of Infarct Size

The infarct size was determined by staining with 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) and was analyzed with image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD). Briefly, 24 h after reperfusion, the mice were sacrificed and the brain was removed and cut into five coronal slices with a brain-cutting matrix (ASI Instruments, Warren, MI). Then the five slices were bathed in TTC solution at 37°C for 30 min, fixed in 4% paraformaldehyde, and photographed with a digital camera. Infarct volume was calculated from the 5 slices using the following formula: infarct volume = (red area of contralateral side − red area of ipsilateral side) / total area × 100% [19].

Cell Culture and Oxygen Glucose Deprivation/Reoxygenation

Primary neurons were obtained from the cerebral cortex of

ICR mouse embryos at E12, E16, and E20 as described previously[20]. The cells were subjected to oxygen glucose deprivation (OGD)/reoxygenation or treated with H_2O_2 on day 8 in culture. Briefly, primary neurons (8 days) were rinsed 3 times with PBS and incubated with glucose-free Hank's balanced salt solution (HBSS; in mmol/L: 151 NaCl, 3.5 $CaCl_2$, 4.8 KCl, 44 HEPES, 7.8 $NaHCO_3$, 1 KH_2CO_3, 2.7 $MgSO_4$, pH 7.4) and placed in a chamber (MC-101, Billups-Rothenberg Inc., San Diego, CA) filled with 95% N_2 and 5% CO_2 at 37°C (for OGD). Control cells were placed in HBSS containing 3 mmol/L *D*-glucose and incubated under normal culture conditions for the same time period. After OGD for 4 h, culture medium was replaced with normal Neurobasal medium (Gibco by Life Technologies, Invitrogen) and cultured under normal conditions for 24 h (for reoxygenation).

Western Blot Analysis

Protein concentrations in extracts from mouse cerebral cortex and primary neurons were determined with the bicinchoninic acid assay (Pierce, Rockford, IL). A total of 70 μg protein was separated *via* electrophoresis on a 10% or 12% sodium dodecyl sulfate polyacrylamide gel, depending on the main protein of interest. Protein was then transferred to nitrocellulose membranes (Millipore, Billerica, MA) in transfer buffer (in mmol/L: 25 Tris base, 192 glycine, 10% [*v*/*v*] methanol). The membranes were blocked for 1 h at room temperature in blocking solution [4% bovine serum albumin (BSA), 0.2% Tween-20 in Tris-buffered saline, pH 7.4]. After blocking, the membranes were incubated overnight with primary antibody diluted in primary antibody buffer (1% BSA, 0.2% Tween-20 in TBS, pH 7.4). The following primary antibodies (and their dilutions) were used: mouse anti-actin (Sigma; 1:5 000), rabbit anti-TIGAR (Sigma; 1:1 000), rabbit anti-γ-H2A.X (Abcam; 1:1 000), rabbit anti-cytochrome *c* (Abcam; 1:1 000), rabbit anti-NOX 4 (Santa Cruz; 1:1 000) and rabbit anti-nitrotyrosine (Millipore; 1:500). Then the membranes were washed 3 × 10 min in TBS and incubated for 1 h at room temperature with appropriate secondary antibody diluted (1:10 000) in secondary antibody buffer (0.5% BSA, 0.2% Tween-20 in TBS, pH 7.4). After three washes, the protein bands were developed with an enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ). Films were scanned and densitometric analyses were performed using AlphaEase software (Alpha Innotech, San Leandro, CA). Samples were normalized to actin and presented as fold of the control group.

Immunofluorescence

Immunofluorescence was carried out as described previously[21] to assess TIGAR expression in mouse brains or primary neurons at different ages. Briefly, frozen brain sections (10 μm) were cut on a cryostat (Leica, Nussloch, Germany). Then the slides or primary neurons were fixed with 4% paraformaldehyde, blocked in 1% normal BSA, and incubated overnight with the primary antibodies anti-TIGAR (1:400), MAP-2 (Sigma; 1:200), GFAP (Sigma; 1:400), γ-H2A.X (1:1 000), or cytochrome *c* (1:500). Then the slices were washed and incubated with secondary antibody. For mitochondrial staining, the cells were incubated with MitoTracker probes (Invitrogen) (1:1 000), which passively diffuse across the plasma membrane and accumulate in active mitochondria. Cell images were acquired with a laser confocal microscope (Carl Zeiss, Oberkochen, Germany).

NADPH Treatment

Primary neurons (8 days) were washed 3 times with PBS and then incubated with Neurobasal medium containing 40 μmol/L NADPH for 4 h. Then the neurons were subjected to OGD for 4 h in HBSS containing 40 μmol/L NADPH. After that, neurons were re-placed in Neurobasal medium containing 40 μmol/L NADPH and cultured under normal conditions for 24 h (reoxygenation). Control cells were maintained in normal conditions.

H_2O_2 Treatment

Primary neurons (8 days) were rinsed 3 times with PBS and incubated with Neurobasal medium containing 720 μmol/L H_2O_2 for 3 h. Control cells were placed in normal medium.

Cell Viability Assay

The viability of cultured primary neurons treated with H_2O_2 was evaluated with a non-radioactive cell-counting kit (CCK-8; Dojindo, Kumamoto, Japan).

DHE Staining for ROS

Neurons were incubated with dihydroethidium (DHE) (1:2000; Beyotime, Nanjing, China) at 37°C for 20 min. Then they were visualized with a confocal microscope (LSM 710, Carl Zeiss, Germany) and fluorescence was measured with a 590 nm filter. Images were stored digitally.

Measurement of NADPH/NADP$^+$ and GSH/GSSG Levels

The levels of NADPH/NADP$^+$ and GSH/GSSG in cerebral cortices and primary neurons were determined 0, 3 and 24 h after ischemia/reperfusion or OGD/reoxygenation with the Enzychrom NADPH/NADP$^+$ assay kit (BioAssay Systems, Hayward, CA) and the GSH/GSSG kit (Beyotime) following the manufacturers' instructions.

Measurement of Lactic Acid Level

The lactic acid (LD) levels in cerebral cortices and primary neurons were determined 0, 3, and 24 h after ischemia/reperfusion or OGD/reoxygenation with an LD assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions.

Single-cell Gel Electrophoresis (Comet) Assay

DNA damage in primary neurons was analyzed with an alkaline comet assay[22]. Using processed cells (3 h after ischemia/reperfusion), 10 μL of cell suspension was added to 90 μL low melting-point agarose. The mixture was placed on a slide containing a normal agarose layer. After solidification (5 min at 4°C), the slides were placed in lysis solution (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, 1% sodium sarcosinate, 1% Triton X-100, and 10% DMSO) for 2 h at 4°C to remove the proteins. After cell lysis and protein extraction, the slides were placed in the tray containing alkaline electrophoresis buffer (1 mmol/L EDTA and 300 mmol/L NaOH, pH 12) for 30 min at 4°C in the dark. Then the slides were subjected to electrophoresis (25 V and 300 mA) for 30 min. After electrophoresis, the slides were neutralized 3 × 5 min with Tris buffer (pH 7.5) and fixed in anhydrous ethanol for 10 min, after which they were stored for subsequent analysis under a fluorescence microscope (Carl Zeiss, Germany). About 100 randomly-selected comets were analyzed on each slide. Images were processed using CASP, a tool for image analysis in comet assay. The data from CASP analysis were plotted using GraphPad Prism5.

Isolation of Mitochondria from Primary Neurons and Cerebral Cortex

After reperfusion or reoxygenation for 24 h, brain tissues from the whole ischemic cortex (infarct and penumbra tissues) or cultured primary neurons were collected for purification of mitochondria using a cell mitochondria isolation kit (Beyotime) and a tissue mitochondria isolation kit (Beyotime) according to the manufacturer's instructions.

Statistical Analysis

Data are presented as mean ± SEM and were analyzed with one-way analysis of variance (ANOVA) with *post hoc* Bonferroni multiple comparisons or two-tailed Student's *t*-test. A difference was considered statistically significant when $P < 0.05$.

RESULTS

TIGAR Level Decreased during Development and Aging

The protein level of TIGAR in cerebral cortex was highest at embryonic day 10, decreased slightly thereafter and robustly dropped at birth. This may be responsible for the susceptibility of neonatal mice to ROS insult[5]. The level partially recovered at postnatal days 4–7 followed by a gradual decline to a low level in early adulthood (Fig. 1A, B). We further compared the TIGAR levels in the brains of 1-month-old and 12-month-old mice. The results showed that the level decreased further in 12-month-old mice (Fig. 1C, D). A similar pattern of developmental changes in TIGAR expression was also seen with immunofluorescence (Fig. 1E). Direct staining for TIGAR and MAP-2 indicated that TIGAR immunofluorescence activity was higher at postnatal day 7 than at postnatal day 4 (Fig. 1F), while double staining for TIGAR and GFAP did not show any difference in TIGAR expression between these ages (Fig. 1G).

Higher Levels of TIGAR Correlated with Greater Tolerance of Neurons to Ischemia/Reperfusion Insult

To determine whether high levels of TIGAR are related to the resistance of neurons to ischemia/reperfusion insult, we examined the changes of TIGAR in mouse brain after ischemia/reperfusion at different ages. TIGAR expression was increased after ischemia/reperfusion at 2 and 3 months. However, in 1-month-old mice, TIGAR expression was significantly decreased 3 h after reperfusion. Therefore, although the basal levels were dissimilar, TIGAR protein levels in these 3 age-groups were maintained at a similar stable level after reperfusion (Fig. 2A, B). However, the 1-month-old group with the highest basal level of TIGAR had the smallest infarct volume (Fig. 2C, D), as well

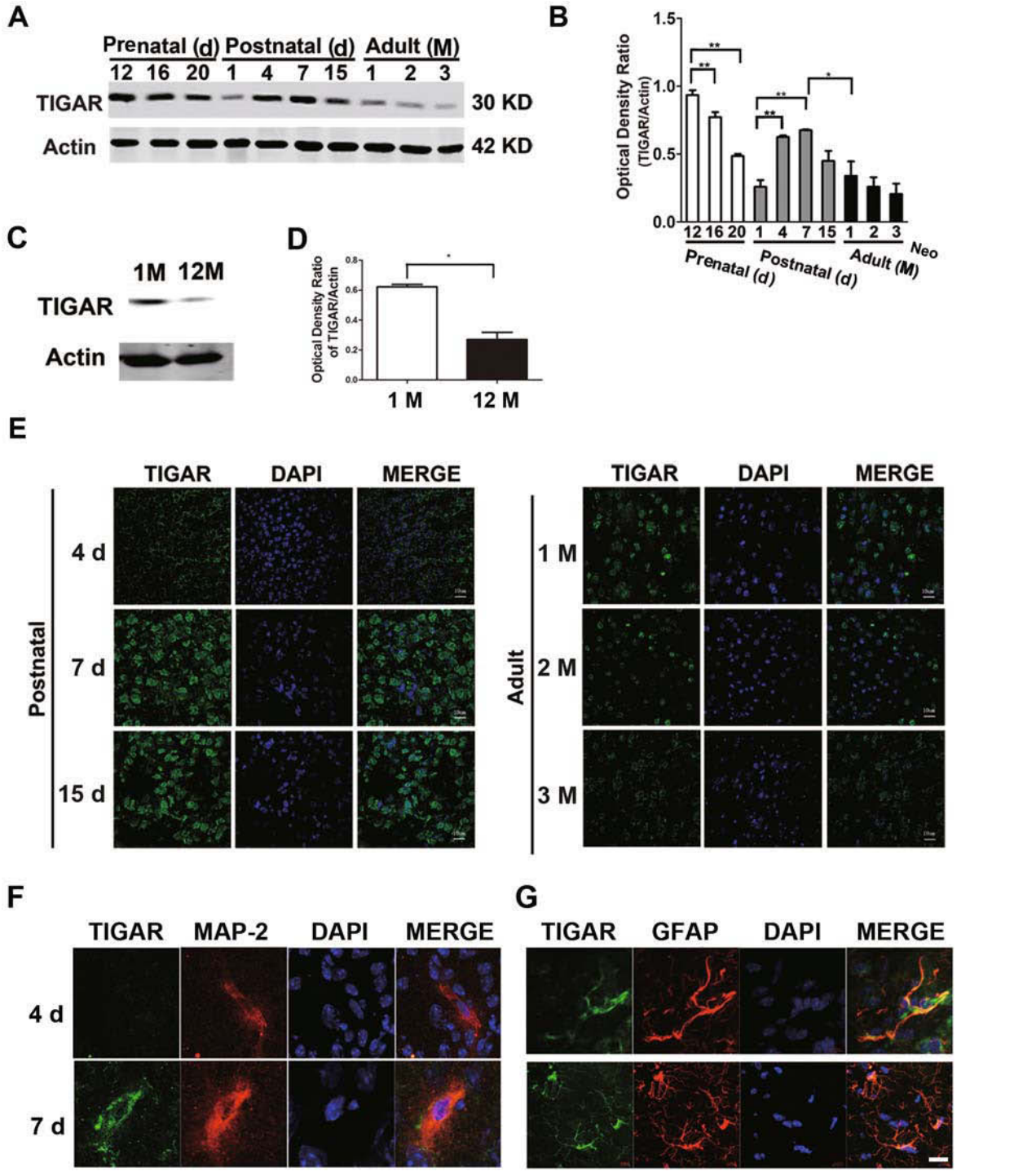

Fig. 1. TIGAR expression during development. (A, B) Developmental changes of TIGAR protein in mouse cerebral cortex. Data are expressed as mean ± SEM. One-way ANOVA with Bonferroni *post hoc* test, $^{*}P$ <0.05, $^{**}P$ <0.01, n = 3. (C, D) Expression levels of TIGAR in young and old mouse brains. Two-tailed Student's *t*-test, $^{*}P$ <0.05, n = 3. (E) Immunofluorescence of TIAGR in developing mouse brain. (F) Brain sections from one-month-old and 12-month-old mice stained with anti-TIGAR and the neuronal marker MAP-2. (G) Brain sections from one-month-old and 12-month-old mice stained with anti-TIGAR and the astrocyte marker GFAP. Scale bar, 10 μm. M: month(s).

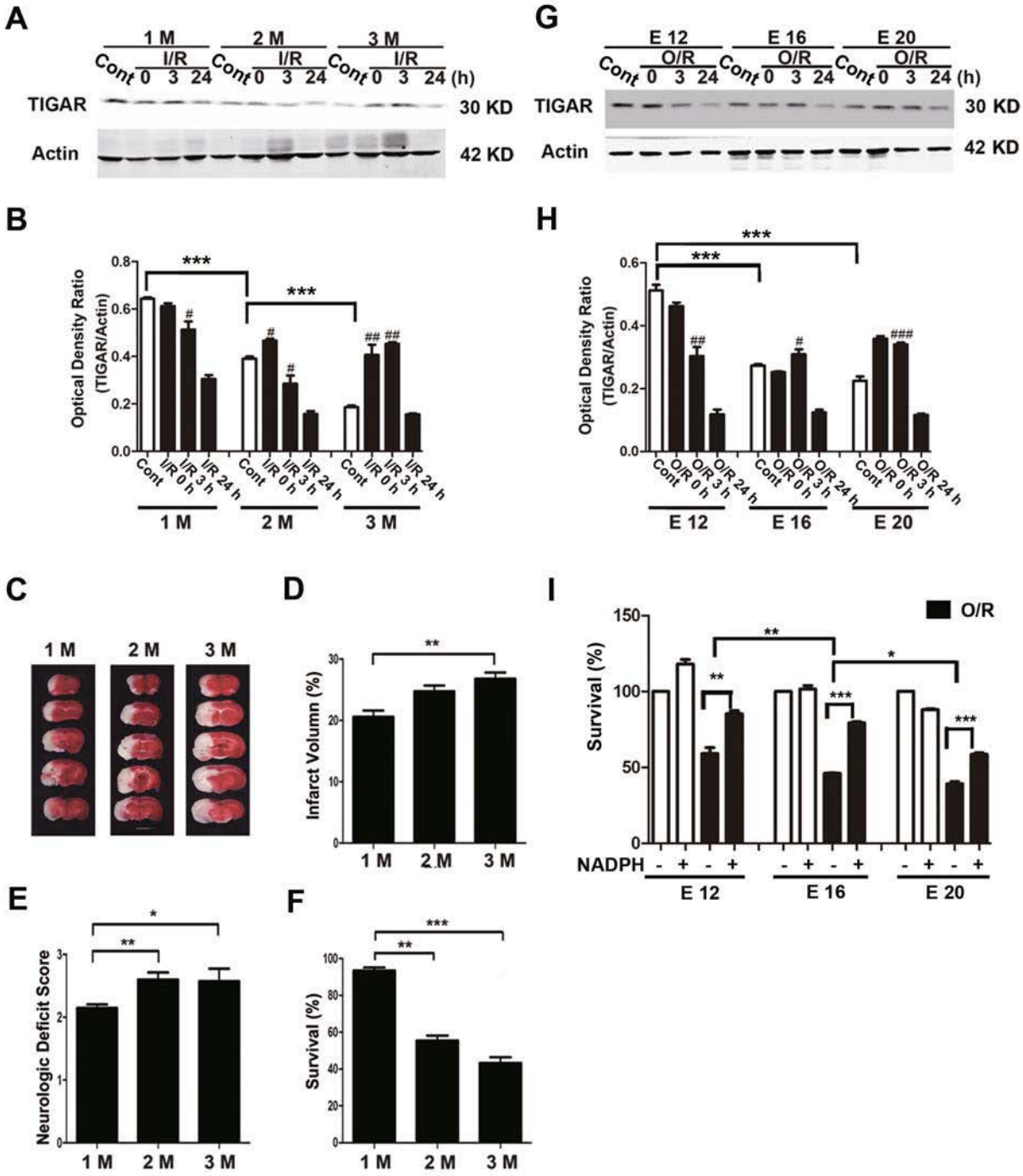

Fig. 2. Upregulation of TIGAR after ischemia/reperfusion *in vitro* and *in vivo*. (A, B) Changes in TIGAR expression in mouse cerebral cortex after ischemia/reperfusion (n = 3; $^{***}P$ <0.001; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs* cont; one-way ANOVA with Bonferroni *post hoc* test). (C) TTC staining of mouse brains subjected to ischemia/reperfusion at 1, 2, and 3 months. (D–F) Quantification of infarct size (D), neurological score (E), and survival rate (F) after ischemia/reperfusion (n = 30; $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001; one-way ANOVA with Bonferroni *post hoc* test). (G, H) Changes in TIGAR expression in primary neurons subjected to OGD/reperfusion (n = 3; $^{***}P$ <0.001; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001 *vs* cont; one-way ANOVA with Bonferroni *post hoc* test). (I) Effects of NADPH on viability of neurons subjected to OGD/reoxygenation (mean ± SEM; n = 3; $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001; one-way ANOVA with Bonferroni *post hoc* test). 1 M, 2 M, 3 M: 1, 2, and 3-month-old mice; E 12, E 16, E 20: embryonic days 12, 16, and 20.

as the lowest neurologic deficit score (Fig. 2E) and death rate compared with the 2- and 3-month-old groups (Fig. 2F).

We also investigated the expression levels of TIGAR in primary neurons from different embryonic ages after reperfusion. The same pattern of changes occurred in primary neurons that underwent OGD/reoxygenation insult (Fig. 2G, H). The viability of primary neurons cultured from embryonic day 12 after OGD/reoxygenation was higher than that of neurons from embryonic days 16 and 20. Supplementation of the primary neurons with NADPH rescued the viability in all three groups (Fig. 2I). These results suggested that TIGAR is a critical modulator of OGD/reoxygenation-induced neuronal death.

Higher Levels of TIGAR Correlated with Greater Tolerance of Neurons to Oxidative Stress

H_2O_2 causes a dose- and time-dependent decrease in cellular viability[23]. To further explore the role of TIGAR in oxidative stress, primary neurons cultured from mice at different embryonic ages were treated with 720 μmol/L H_2O_2 for 3 h to simulate oxidative stress. The results demonstrated that TIGAR expression was up-regulated in neurons cultured from day 16 and 20 embryos. Again, TIGAR expression changed to similar levels in the three groups under oxidative stress (Fig. 3A, B). After H_2O_2 treatment, the neurons with highest basal level of TIGAR (E12) had the highest survival rate (Fig. 3C).

To investigate the potential mechanism by which TIGAR protects against ischemia/reperfusion injury, ROS production in primary neurons from mice of different embryonic ages were determined with DHE staining. All three groups had increased ROS levels after H_2O_2 treatment. The neurons from embryonic day 20 showed a greater increase in ROS levels than those from embryonic day 12 (Fig. 3D, E).

TIGAR Level Correlated with Pentose Phosphate Pathway Activity

To determine whether the responses of cellular redox homeostasis to ischemia vary with age, we measured the levels of NADPH/$NADP^+$, GSH/GSSG, and LD *in vivo* and *in vitro* and found that they differed among different age groups. The highest levels of NADPH (Fig. 4A) and GSH (Fig. 4B), and the lowest levels of LD (Fig. 4C) were found in 1-month-old mice. The NADPH (Fig. 4A) and GSH levels (Fig. 4B) were markedly decreased immediately after reperfusion (0 h), but appeared to partially recover at 3 h. The pattern of change for NADPH and GSH after ischemia/reperfusion injury did not significantly differ among groups. The LD levels quickly increased at the onset of reperfusion (Fig. 4C) and were inversely correlated with the TIGAR levels. Similar results were obtained in primary cultured cortical neurons (Fig. 4D, E). Higher basal levels of LD occurred in neurons from day 16 and 20 embryos compared to those from day 12 embryos. LD increased sharply in all three groups during reoxygenation (Fig. 4F).

We also investigated the correlation between TIGAR expression level and oxidative stress. Higher NADPH levels were found in young than in old mouse brains (Fig. 4G). Furthermore, we compared the oxidative stress in the brains of 1-month-old and 12-month-old mice and found that NOX 4 transiently increased after ischemia, but rapidly declined 3 h after reperfusion in young mouse brains, whereas it was maintained at relative high levels in old mouse brains (Fig. 4H). Nitrotyrosine was up-regulated 3 h after reperfusion in both young and old mouse brains, and was much higher in the old mice.

Increased DNA Damage Response (DDR) to Ischemia/Reperfusion during Development

As previous findings suggested that TIGAR regulates the DDR[24], we examined the γ-H2A.X expression in mouse brain. The result showed that the γ-H2A.X expression significantly increased after 3 h of reperfusion (Fig. 5A, B). Furthermore, the γ-H2A.X expression in older mice (2 and 3 months) was significantly higher than that in younger mice (1 month old) (Fig. 5C, D). Similar results were obtained in cultured neurons after 3 h of reoxygenation (Fig. 5E, F). γ-H2A.X was markedly increased in neurons cultured at different embryonic ages as shown by immunofluorescence. The increase of γ-H2A.X production in the embryonic-day-20 group was most robust (Fig. 5G). We also investigated DNA damage, another biomarker of oxidative stress[25,26]. The data showed that the DNA damage 3 h after OGD was more severe in neurons from later embryonic days (Fig. 5H), as evidenced by increased Comet tail length and DNA content in the tail (Fig. 5I, J). These results suggest that higher TIGAR expression in younger mice may confer greater resistance to ischemic DNA damage.

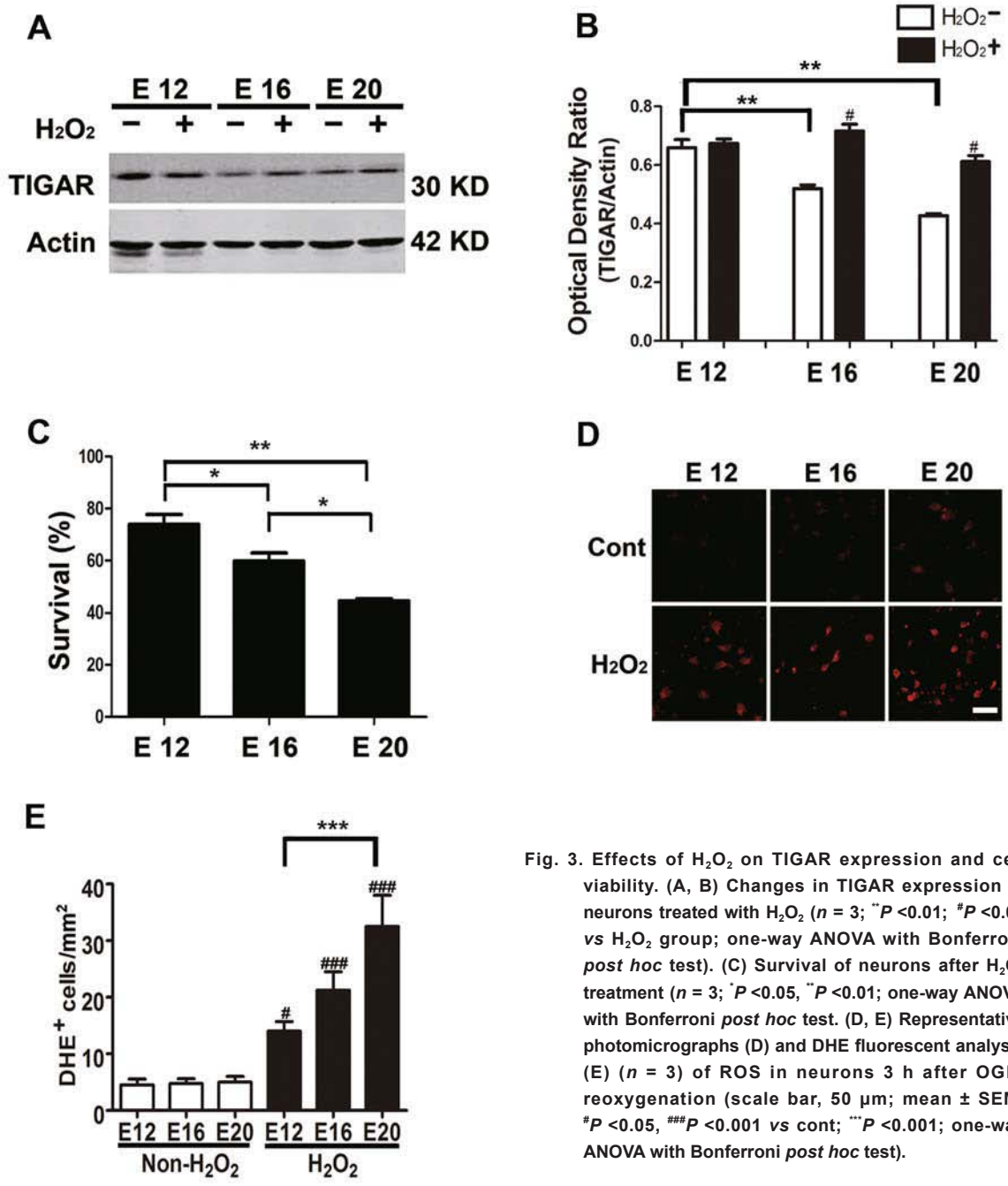

Fig. 3. Effects of H_2O_2 on TIGAR expression and cell viability. (A, B) Changes in TIGAR expression in neurons treated with H_2O_2 (*n* = 3; $^{**}P$ <0.01; $^{\#}P$ <0.05 *vs* H_2O_2 group; one-way ANOVA with Bonferroni *post hoc* test). (C) Survival of neurons after H_2O_2 treatment (*n* = 3; $^{*}P$ <0.05, $^{**}P$ <0.01; one-way ANOVA with Bonferroni *post hoc* test. (D, E) Representative photomicrographs (D) and DHE fluorescent analysis (E) (*n* = 3) of ROS in neurons 3 h after OGD/reoxygenation (scale bar, 50 μm; mean ± SEM; $^{\#}P$ <0.05, $^{\#\#\#}P$ <0.001 *vs* cont; $^{***}P$ <0.001; one-way ANOVA with Bonferroni *post hoc* test).

Ischemia/Reperfusion Induced More Severe Mitochondrial Damage in Aged Mouse Brain

Cytochrome *c* was released to the cytoplasm 6 h after ischemia/reperfusion (Fig. 6A, B), and the release was highest in 3-month-old mice (Fig. 6C, D). Similar results were obtained with immunofluorescence in primary neurons (Fig. 6E). With increasing gestational age, the re-localization of cytochrome *c* from mitochondria to the cytoplasm increased after OGD/reoxygenation insult.

DISCUSSION

In previous study, we demonstrated that TIGAR is upregulated in response to ischemia/reperfusion, and manipulation of TIGAR levels significantly alters the

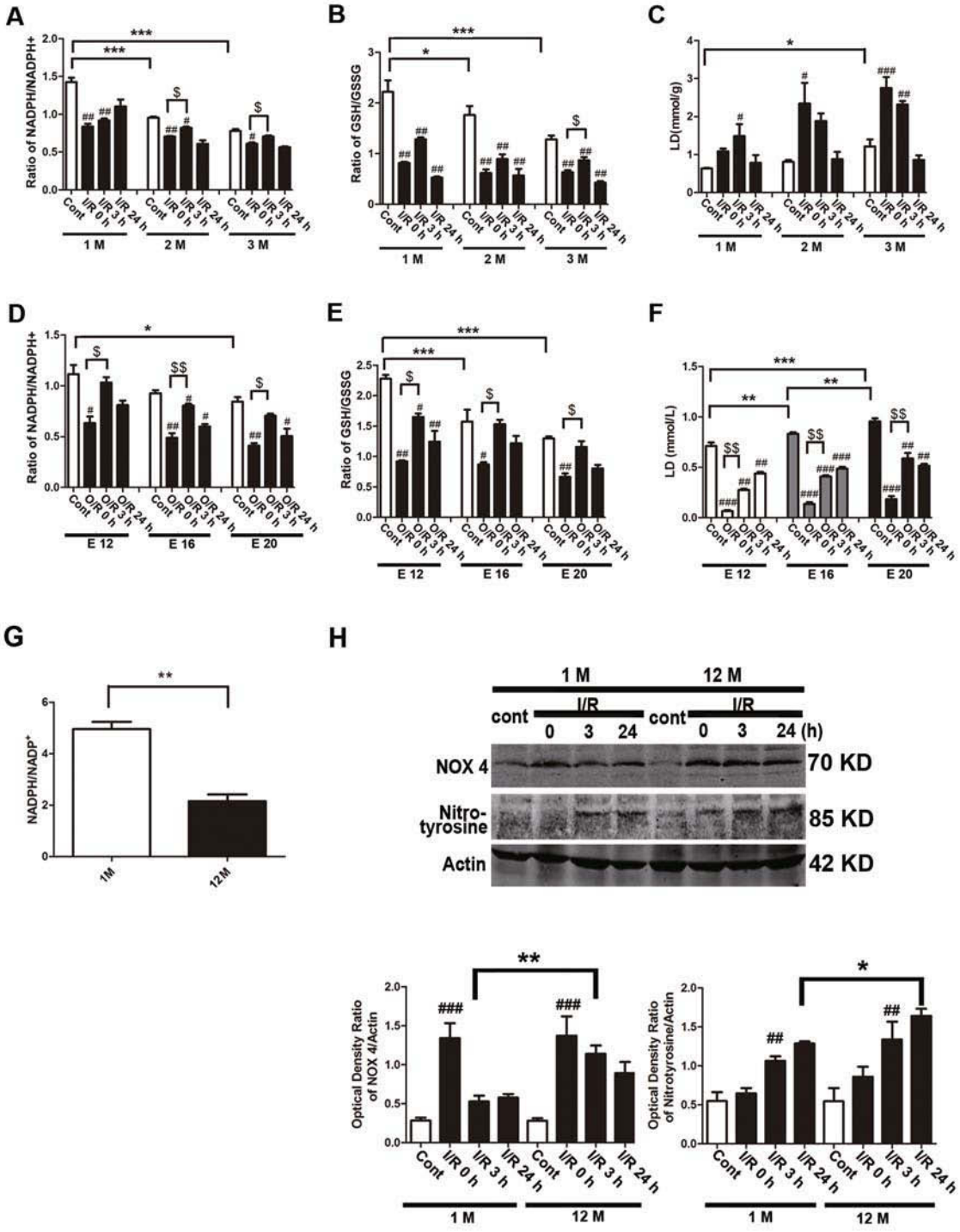

Fig. 4. PPP activity in mice and primary neurons at different ages. (A–C) Levels of NADPH/NADP$^+$ (A), GSH/GSSG (B), and LD (C) in mouse cerebral cortex at 1, 2, and 3 months. (D–F) Levels of NADPH/NADP$^+$ (D), GSH/GSSG (E), and LD (F) in primary neurons from embryonic days 12, 16, and 20. In A–F: n = 5; $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001 *vs* cont; $^{\$}P$ <0.05, $^{\$\$}P$ <0.01; one-way ANOVA with Bonferroni *post hoc* test. (G) Comparison of NADPH/NADP$^+$ levels in young and old mouse brain (n = 3; $^{**}P$ <0.01; two-tailed Student's t-test). (H) NOX4 and nitrotyrosine after reperfusion in 1-month and 12-month-old mouse brain (n = 3; mean ± SEM; $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001 *vs* cont; $^{*}P$ <0.05, $^{**}P$ <0.01; one-way ANOVA with Bonferroni *post hoc* test). M: month(s).

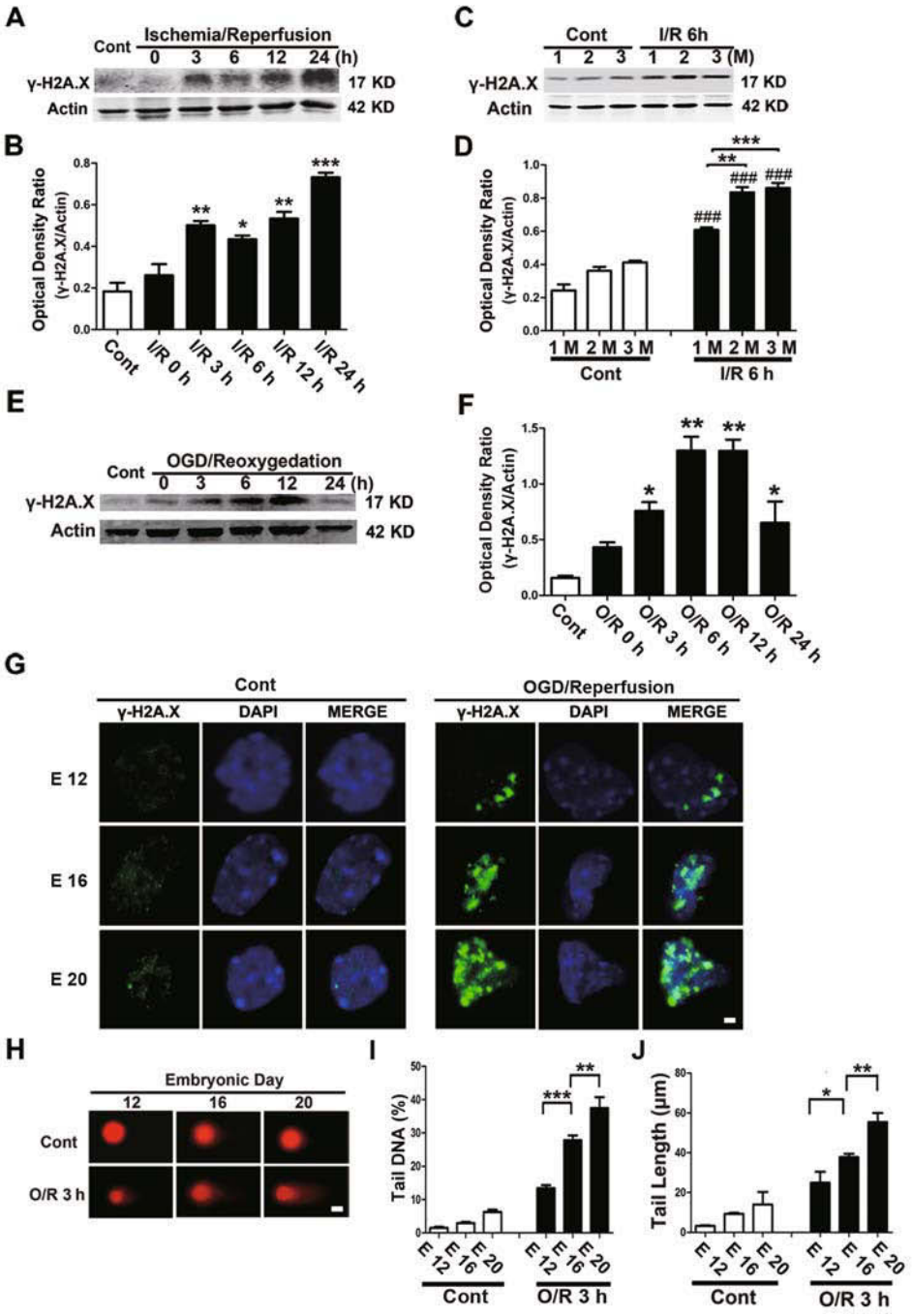

Fig. 5. DNA damage in mouse brains and primary neurons. (A, B) Time-course of changes in γ-H2A.X in mouse cerebral cortices after ischemia/reperfusion (*n* = 3; $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$< 0.001; one-way ANOVA with Bonferroni *post hoc* test). (C, D) Upregulation of γ-H2A.X in cerebral cortices of mice at different ages 3 h after ischemia/reperfusion (*n* = 3; $^{\#\#\#}P$ <0.001 *vs* cont; $^{**}P$ <0.01, $^{***}P$ <0.001; one-way ANOVA with Bonferroni *post hoc* test). (E, F) Time-course of changes of γ-H2A.X in primary neurons 3 h after reperfusion (*n* = 3; $^{*}P$ <0.05, $^{**}P$ <0.01 *vs* cont; one-way ANOVA with Bonferroni *post hoc* test). (G) Representative immunofluorescent image of γ-H2A.X in primary neurons from different embryonic days (scale bar, 10 μm). (H–J) Comet assays. Primary neurons from different embryonic days were subjected to OGD/reperfusion. Images showing DNA damage (H) (scale bar, 10 μm) and statistics of comet tail length (I) and comet tail DNA content (J) after 3 h of reoxygenation (*n* = 3; mean ± SEM; $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001; one-way ANOVA with Bonferroni *post hoc* test.

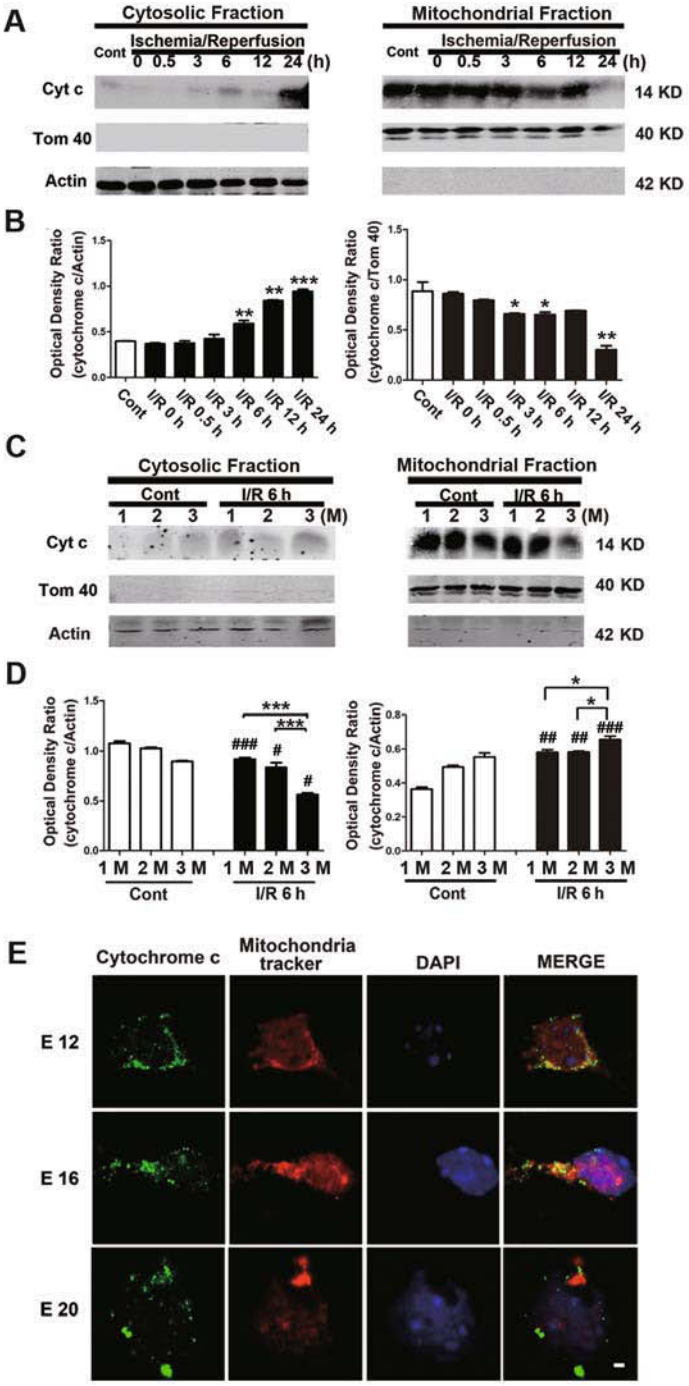

Fig. 6. Mitochondrial damage in mouse cerebral cortices and primary neurons after ischemia/reperfusion. (A, B) Time-course of cytochrome *c* release in mouse cerebral cortices after ischemia/reperfusion (n = 3; $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001 *vs* cont, one-way ANOVA with Bonferroni *post hoc* test). (C, D) Cytochrome *c* release in cerebral cortices of mice after ischemia/reperfusion insult at different ages (n = 3; mean ± SEM; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01, $^{\#\#\#}P$ <0.001 *vs* cont; $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001; one-way ANOVA with Bonferroni *post hoc* test). (E) Representative double immunofluorescent images of cytochrome *c* and mitochondrial tracker in primary neurons cultured on different embryonic days. Loss of mitochondrial cytochrome *c* in neurons from embryonic day 20 was much greater than that in neurons from earlier embryonic ages (scale bar, 10 μm).

sensitivity of neurons to ischemia/reperfusion damage and long-term post-ischemia survival and functional recovery. In the present study, we found higher levels of TIGAR in neonatal cerebral cortex and neurons of earlier embryonic age. This pattern of TIGAR expression was correlated with the sensitivity of neurons to ischemia/reperfusion insult. Young adult mice with higher basal TIGAR protein levels had smaller infarct volumes, as well as lower neurological deficit scores and death rates after ischemia/reperfusion. Similarly, primary neurons cultured at an earlier embryonic age had significantly higher basal levels of TIGAR protein, and this resulted in higher neuronal survival rates after OGD/reoxygenation. Primary neurons with higher basal levels of TIGAR were also more resistant to H_2O_2-induced neuronal damage. Neurons cultured at an earlier embryonic age had lower levels of ROS after H_2O_2 treatment than those at a later age. These results suggest that the expression level of TIGAR is correlated to the vulnerability of neurons to ischemic insult *in vivo* and *in vitro*. It should be pointed out that many other factors contribute to the differing vulnerability of neurons to ischemic injury during development and maturation[28-31]. For example, many studies have reported that the expression of NMDA receptor subunits changes during neuronal development[28,32]. The changes in NMDA receptor number and functionality during neuronal development and maturation may also account for the differing vulnerability of neurons to ischemic injury.

It is now well-established that NADPH derived from the PPP is a key molecule in maintaining the function of several important redox and antioxidant defense mechanisms through the production of GSH[33,34], a cysteine-containing tripeptide essential for maintaining the normal reduced state of cells[35,36]. Oxygen deprivation induces a rapid stimulation of glycolysis, the well-known Pasteur effect[37]. However, neurons have little capacity to perform anaerobic glycolysis due to the low activity of Pfkfb3 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3)[38]. We found that the high expression of TIGAR in younger adult mice and primary neurons from earlier embryonic periods had higher NADPH/NADP+ and GSH/GSSG levels under basal conditions. However, the pattern of GSH levels did not change after ischemia/reperfusion. The basal LD (the product of glycolysis) level in mice was inversely correlated with the expression level of TIGAR, higher TIGAR levels resulting in lower LD levels. After ischemia/reperfusion, the increase in LD levels was greater in mice with lower TIGAR levels. However, in cultured primary neurons, there was no such correlation. Ischemia/reperfusion also induced much more severe oxidative stress in the brains of old mice than in young mice after ischemic insult. We also found that the TIGAR level was robustly elevated in the young adult mouse brain after ischemia/reperfusion and in primary neurons from later embryonic mouse brains after OGD/reoxygenation. These are consistent with the previous report that TIGAR inhibits the glycolytic rate and promotes PPP flow, actions that favor neuronal survival under ischemic conditions[15].

However, TIGAR did not change or even decreased in younger adult mouse brains and in primary neurons cultured from earlier embryonic ages. This differential change brought the TIGAR level almost to the same level among the different-aged groups after ischemic insult. Thus, the vulnerability of neurons to ischemic damage appeared to correlate closely with the basal level of TIGAR. On the other hand, why neurons from various developmental stages respond differently is not clear.

Numerous studies have demonstrated the role of ROS in the pathophysiology of neurological disorders, including ischemia and degenerative diseases[39]. ROS cause macromolecular damage such as DNA oxidation, which can lead to cell injury and death[40]. ROS accumulate as byproducts of the electron-transport chain, causing mitochondrial damage[41]. TIGAR contributes to the regulation of intracellular ROS levels by modulating the glycolytic pathway[13,37]. In this study, we found that ischemia/reperfusion and OGD/reoxygenation induced severe DNA damage in mouse neurons. The lower TIGAR expression in older adult mouse brains and neurons cultured from later embryonic ages aggravated the DNA and mitochondrial damage, when compared to younger adult mouse brains and neurons cultured from an earlier embryonic period. These data suggested that younger adult mice and neurons from an earlier embryonic period have a stronger ability to switch the metabolic pathway from glycolysis to PPP and to reduce oxidative stress.

In summary, the basal level of TIGAR was higher in young adult brains and early embryonic neurons. There was a correlation between the PPP activity and anti-

oxidation capability. These data suggest that the decline in the expression level of TIGAR is correlated with the age-dependent increase in vulnerability of neurons to ischemic injury and may be a new therapeutic target for stroke.

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REFERENCES

[1] Lui VW, Wong EY, Ho K, Ng PK, Lau CP, Tsui SK, *et al*. Inhibition of c-Met downregulates TIGAR expression and reduces NADPH production leading to cell death. Oncogene 2011, 30: 1127–1134.

[2] Crack PJ, Taylor JM. Reactive oxygen species and the modulation of stroke. Free Radic Biol Med 2005, 38: 1433–1444.

[3] Niizuma K, Endo H, Chan PH. Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. J Neurochem 2009, 109 Suppl 1: 133–138.

[4] Shakil H, Saleem S. Genetic deletion of prostacyclin IP receptor exacerbates transient global cerebral ischemia in aging mice. Brain Sci 2013, 3: 1095–1108.

[5] Sheldon RA, Jiang X, Francisco C, Christen S, Vexler ZS, Tauber MG, *et al*. Manipulation of antioxidant pathways in neonatal murine brain. Pediatr Res 2004, 56: 656–662.

[6] Kratzer I, Chip S, Vexler ZS. Barrier mechanisms in neonatal stroke. Front Neurosci 2014, 8: 359.

[7] Bentsen L, Christensen L, Christensen A, Christensen H. Outcome and risk factors presented in old patients above 80 years of age versus younger patients after ischemic stroke. J Stroke Cerebrovasc Dis 2014, 23: 1944–1948.

[8] Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. Cell 2005, 120: 483–495.

[9] Chistiakov DA, Sobenin IA, Revin VV, Orekhov AN, Bobryshev YV. Mitochondrial aging and age-related dysfunction of mitochondria. Biomed Res Int 2014, 2014: 238463.

[10] Vajapey R, Rini D, Walston J, Abadir P. The impact of age-related dysregulation of the angiotensin system on mitochondrial redox balance. Front Physiol 2014, 5: 439.

[11] Korzick DH, Lancaster TS. Age-related differences in cardiac ischemia-reperfusion injury: effects of estrogen deficiency. Pflugers Arch 2013, 465: 669–685.

[12] Trocha M, Merwid-Lad A, Chlebda-Sieragowska E, Szuba A, Piesniewska M, Fereniec-Golebiewska L, *et al*. Age-related changes in ADMA-DDAH-NO pathway in rat liver subjected to partial ischemia followed by global reperfusion. Exp Gerontol 2014, 50: 45–51.

[13] Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, *et al*. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 2006, 126: 107–120.

[14] Okar D.A, Manzano A, Navarro-Sabate A, Riera L, Bartrons R, Lange AJ. PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem Sci 2001, 26: 30–35.

[15] Li M, Sun M, Cao L, Gu JH, Ge J, Chen J, *et al*. A TIGAR-regulated metabolic pathway is critical for protection of brain ischemia. J Neurosci 2014, 34: 7458–7471.

[16] Ghosh D, Levault KR, Brewer GJ. Relative importance of redox buffers GSH and NAD(P)H in age-related neurodegeneration and Alzheimer disease-like mouse neurons. Aging Cell 2014, 13: 631–640.

[17] Clark WM, Lessov NS, Dixon MP, Eckenstein F. Monofilament intraluminal middle cerebral artery occlusion in the mouse. Neurol Res 1997, 19: 641–648.

[18] Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989, 20: 84–91.

[19] Sheng R, Zhang LS, Han R, Liu XQ, Gao B, Qin ZH. Autophagy activation is associated with neuroprotection in a rat model of focal cerebral ischemic preconditioning. Autophagy 2010, 6: 482–494.

[20] Yonekura I, Takai K, Asai A, Kawahara N, Kirino T. p53 potentiates hippocampal neuronal death caused by global ischemia. J Cereb Blood Flow Metab 2006, 26: 1332–1340.

[21] Tamatani M, Matsuyama T, Yamaguchi A, Mitsuda N, Tsukamoto Y, Taniguchi M, *et al*. ORP150 protects against hypoxia/ischemia-induced neuronal death. Nat Med 2001, 7: 317–323.

[22] Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988, 175: 184–191.

[23] Garg TK, Chang JY. 15-deoxy-delta 12, 14-Prostaglandin J2 prevents reactive oxygen species generation and mitochondrial membrane depolarization induced by oxidative stress. BMC Pharmacol 2004, 4: 6.

[24] Sinha S, Ghildiyal R, Mehta VS, Sen E. ATM-NFkappaB axis-driven TIGAR regulates sensitivity of glioma cells to radiomimetics in the presence of TNFalpha. Cell Death Dis 2013, 4: e615.

[25] Gupta S, Yel L, Kim D, Kim C, Chiplunkar S, Gollapudi S.

Arsenic trioxide induces apoptosis in peripheral blood T lymphocyte subsets by inducing oxidative stress: a role of Bcl-2. Mol Cancer Ther 2003, 2: 711–719.

[26] Kumar S, Yedjou CG, Tchounwou PB. Arsenic trioxide induces oxidative stress, DNA damage, and mitochondrial pathway of apoptosis in human leukemia (HL-60) cells. J Exp Clin Cancer Res 2014, 33: 42.

[27] Sugawara T, Fujimura M, Morita-Fujimura Y, Kawase M, Chan PH. Mitochondrial release of cytochrome c corresponds to the selective vulnerability of hippocampal CA1 neurons in rats after transient global cerebral ischemia. J Neurosci 1999, 19: RC39.

[28] Menezes FP, Kist LW, Bogo MR, Bonan CD, Da Silva RS. Evaluation of age-dependent response to NMDA receptor antagonism in zebrafish. Zebrafish 2015, 12: 137–143.

[29] Thompson JW, Narayanan SV, Koronowski KB, Morris-Blanco K, Dave KR, Perez-Pinzon MA. Signaling pathways leading to ischemic mitochondrial neuroprotection. J Bioenerg Biomembr 2015, 47: 101–110.

[30] Cekanaviciute E, Fathali N, Doyle KP, Williams AM, Han J, Buckwalter MS. Astrocytic transforming growth factor-beta signaling reduces subacute neuroinflammation after stroke in mice. Glia 2014, 62: 1227–1240.

[31] Quillinan N, Grewal H, Deng G, Shimizu K, Yonchek JC, Strnad F, *et al*. Region-specific role for GluN2B-containing NMDA receptors in injury to Purkinje cells and CA1 neurons following global cerebral ischemia. Neuroscience 2015, 284: 555–565.

[32] Celso Constantino L, Tasca CI, Boeck CR. The role of NMDA receptors in the development of brain resistance through pre- and postconditioning. Aging Dis 2014, 5: 430–441.

[33] Martensson J, Lai JC, Meister A. High-affinity transport of glutathione is part of a multicomponent system essential for mitochondrial function. Proc Natl Acad Sci U S A 1990, 87: 7185–7189.

[34] Griffith OW, Meister A. Origin and turnover of mitochondrial glutathione. Proc Natl Acad Sci U S A 1985, 82: 4668–4672.

[35] Gupte SA, Arshad M, Viola S, Kaminski PM, Ungvari Z, Rabbani G, *et al*. Pentose phosphate pathway coordinates multiple redox-controlled relaxing mechanisms in bovine coronary arteries. Am J Physiol Heart Circ Physiol 2003, 285: H2316–2326.

[36] Gupte SA, Rupawalla T, Phillibert D Jr, Wolin MS. NADPH and heme redox modulate pulmonary artery relaxation and guanylate cyclase activation by NO. Am J Physiol 1999, 277: L1124–1132.

[37] Vanoverschelde JL, Janier MF, Bakke JE, Marshall DR, Bergmann SR. Rate of glycolysis during ischemia determines extent of ischemic injury and functional recovery after reperfusion. Am J Physiol 1994, 267: H1785–1794.

[38] Almeida A, Moncada S, Bolanos JP. Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. Nat Cell Biol 2004, 6: 45–51.

[39] Chan PH. Oxygen radicals in focal cerebral ischemia. Brain Pathol 1994, 4: 59–65.

[40] Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cereb Blood Flow Metab 2001, 21: 2–14.

[41] Andreyev AY, Kushnareva YE, Starkov AA. Mitochondrial metabolism of reactive oxygen species. Biochemistry (Mosc) 2005, 70: 200–214.